The Journal of Neuroscience, February 1995, 15(2): 1180–1194

Enhanced Survival and Neuronal Differentiation of Adrenal Chromaffin Cells Cografted into the Striatum with NGF-producing Fibroblasts

Kenji Niijima,[1] Gordon R. Chalmers,[2] Daniel A. Peterson,[2] Lisa J. Fisher,[2] Paul H. Patterson,[1] and Fred H. Gage[2]

[1]Division of Biology, California Institute of Technology, Pasadena, California 91125 and [2]Department of Neurosciences, School of Medicine, University of California at San Diego, La Jolla, California 92093-0627

Although adrenal medullary chromaffin cells have been used extensively for intracerebral grafting, their survival has generally been poor. Improved survival of the implanted cells has been achieved by exposing the chromaffin cells to NGF *in vivo*. Culture studies have shown, however, that chromaffin cells are converted into sympathetic neurons when NGF is included in the medium. The degree to which such a transdifferentiation may occur *in vivo* has not been determined. We assessed the effects of cografting chromaffin cells with primary fibroblasts genetically engineered to express NGF. Chromaffin cells from 10 d old rats were implanted with NGF-producing or β-galactosidase–producing primary fibroblasts (control fibroblasts) into the striatum of 6-hydroxydopamine treated adult rats of the same strain. Eight weeks postgrafting, chromaffin cells cografted with NGF-producing fibroblasts displayed many of the features of mature sympathetic neurons such as large somata, long processes, transmitter vesicles similar to those found in neurons, and positive immunolabeling for the neuronal markers neurofilament, MAP2 and SCG10. Chromaffin-derived neuron number was also significantly enhanced in the presence of NGF-producing fibroblasts. While control fibroblasts were also found to increase chromaffin cell number above that of chromaffin cells grafted alone, the control fibroblasts did not induce neuronal transdifferentiation. These results demonstrate that chromaffin cells cografted with NGF-producing fibroblasts undergo transdifferentiation *in vivo* and express many characteristics of mature sympathetic neurons. The consequences of this transdifferentiation on the long term survival and function of the transplanted cells *in vivo* remain to be clarified.

[Key words: adrenal chromaffin cells, autograft, NGF, transdifferentiation, genetically engineered fibroblasts]

Chromaffin cells have been widely used in neural grafting studies in animal models of dopamine deficiency (see reviews by Fine, 1990; Freed et al., 1990). Survival of chromaffin cells transplanted into the brain parenchyma is, however, generally poor (Freed et al., 1986; Bohn et al., 1987; Hansen et al., 1988; Hurtig et al., 1989; Jankovic et al., 1989; Peterson et al., 1989; Hirsch et al., 1990; Kordower et al., 1991). To increase the survival of grafted chromaffin cells, supplementation with NGF has been successfully employed (Stromberg et al., 1985; Date et al., 1990; Kordower et al., 1990; Cunningham et al., 1991). In addition to increasing chromaffin cell survival, NGF also induces the chromaffin cells to develop neurites (Stromberg et al., 1985; Date et al., 1990; Kordower et al., 1990; Cunningham et al., 1991). The neuritic outgrowths observed from grafted chromaffin cells after NGF administration suggest that the cells may be transdifferentiating into sympathetic neurons *in vivo*. The adrenal medullary chromaffin cell is a member of the neural-crest derived, sympathoadrenal lineage, that also includes sympathetic principal neurons and small intensely fluorescent (SIF) cells. These cell types are interconvertible to a considerable extent. Chromaffin cells in the rat adrenal medulla are characterized by phenylethanolamine *N*-methyltransferase (PNMT) expression and epinephrine synthesis in 80% of the cells, and large catecholamine storage vesicles and a small cell body size, for example, 20 μm diameter. These characteristics are maintained *in vitro* if glucocorticoids are included in the culture media (Doupe et al., 1985). In the absence of glucocorticoids, cultured chromaffin cells lose their differentiated characteristics and die. In contrast, NGF induces the expression of characteristics of mature sympathetic neurons in postnatal (Unsicker et al., 1978; Doupe et al., 1985) or embryonic chromaffin cells *in vitro* (Anderson and Axel, 1986; Seidl, 1989a,b) and enhances the survival of these cells. These neuronal characteristics include increased cell size, loss of PNMT and epinephrine synthesis, and development of immunoreactivity to neurofilament protein (Doupe et al., 1985). Characteristic neuronal ultrastructure is also acquired, including abundant somal rough endoplasmic reticulum and golgi bodies, loss of somal chromaffin granules, and neurite formation with microtubules, smooth endoplasmic reticulum and varicosities filled with synaptic vesicles.

Except for the formation of neurites, it is unknown to what degree chromaffin cells can transdifferentiate into sympathetic neurons under the influence of NGF *in vivo*. It is known that a sympathoadrenal cell line established from embryonic rat ad-

Received Mar. 21, 1994; revised July 25, 1994; accepted Aug. 2, 1994.

K.N. and G.R.C. contributed equally to this article. This work was supported by NIH AG 10435 and AG 08514, The Margret and Herbert Hoover Foundation, Allied Signal Award and Metropolitan Life Award to F.H.G and Amgen Inc. to P.H.P. K.N. was supported by a CNS fellowship of Eli Lilly Japan K.K. D.A.P. was supported by NIH Fellowship AGO 5512-02. We thank Dr. David J. Anderson for the anti-SCG10 antibody, Dr. Ikuko Nagatsu for the anti-TH antibody, Mr. Ming-Ji Fann for contributions to the early stages of this work, and Ms. Doreen McDowell for media preparation.

Correspondence should be addressed to Dr. Fred Gage at the above address.

renal cells (MAH-B2) is able to express the neuron-specific markers MAP2 and neurofilament when grafted within peripheral nerves (Doering et al., 1992). In the present study, we explored whether it is possible to manipulate the environmental conditions of chromaffin cells *in vivo* to induce the cells to transdifferentiate and survive for 8 weeks. If an implanted chromaffin cell expresses features of a neuron it may be better able to integrate and survive within the environment of the brain. In this way chromaffin cells may have the potential to serve as a source of neurons whose phenotype is under experimental control (Mahanthappa et al., 1990).

We have employed genetically modified fibroblasts in cografts with chromaffin cells to supply the NGF locally (Gage et al., 1991). Fully differentiated chromaffin cells from postnatal day 10 Fischer 344 rats were grafted into the striatum of adult rats (of the same strain) with NGF-producing or control fibroblasts, or without any fibroblasts at all. To determine if chromaffin cell transdifferentiation and survival are influenced by striatal dopaminergic denervation, transplants were implanted into either intact striata or striata that had been denervated by injections of 6-hydroxydopamine (6-OHDA) into the substantia nigra. Eight weeks after grafting, chromaffin cell number was quantified and neuronal transdifferentiation was assessed by measuring the size of cell somata, the production of neurites, immunohistochemical staining with neuron-specific markers, and ultrastructural examination. Chromaffin cells cografted with NGF-producing fibroblasts were found to express a number of characteristics of sympathetic neurons, indicating that *in vivo* transdifferentiation had occurred. In addition, chromaffin-derived neuron number was enhanced by the NGF delivered by genetically modified fibroblasts, and was far greater than that reported previously.

Materials and Methods

Preparation of genetically modified fibroblasts. Primary skin fibroblasts were obtained from a biopsy of the ventral abdominal wall of a female Fischer 344 rat (Harlan Sprague Dawley, Indianapolis IN). These cells were grown in culture with Dulbecco's modified Eagle's medium (DMEM) supplemented with fungizone, L-glutamate, fetal bovine serum, and gentamicin (1, 1, 10, and 0.5 ml/100 ml DMEM, respectively). When 20–30% confluent, fibroblasts were infected with retroviral vectors derived from Moloney murine leukemia virus that contained the cDNA for either mouse β-NGF (Rosenberg et al., 1988) or for β-galactosidase (Bgal). Both vectors also contained the neomycin-resistance gene driven from an internal Rous sarcoma virus promoter. Cells that successfully incorporated the transgene were isolated by growing the cells in the presence of the neomycin analog G418 (400 μg/ml).

Catecholamine lesions. Rats received a unilateral 6-OHDA lesion of the nigrostriatal pathway to remove endogenous dopaminergic innervation of the striatum. The 6-OHDA was dissolved at a concentration of 6 μg/μl in physiological saline containing 0.1% ascorbic acid. Female Fischer 344 rats (130–160 gm) were anesthetized with a mixture of ketamine (75 mg/kg), xylazine (4.0 mg/kg), and acepromazine (5.6 mg/kg) and received 2 μl of 6-OHDA injected unilaterally into the left medial forebrain bundle (AP −4.4 mm, ML 1.1 mm, DV 7.5 mm). Rats were tested with apomorphine (0.1 mg/kg) 7–10 d postlesion and those that displayed greater than 5 rotations/min over a 30 min period after drug administration were selected for grafting.

Preparation of dissociated chromaffin cells. Ten day old Fischer 344 rats (Simonsen Laboratories, Gilroy, CA) were decapitated, the adrenals removed, and the adrenal medullae quickly dissected from the adrenal cortices in ice cold L-15 Air medium (Hawrot and Patterson, 1979). Adrenal medullae were washed several times in L-15 Air medium and then incubated in 0.5 mg/ml collagenase (Worthington Biochemical Co.) and 15 mg/ml dispase (Boehringer Mannheim) in L-15 Air medium for 30 min at 37°C. After removing the collagenase and dispase by a wash, the cells were further incubated in 0.1% trypsin (GIBCO) for 10 min at 37°C. Cells were then washed twice in calcium/magnesium-free Hanks' balanced salt solution (GIBCO) and triturated in the DMEM containing 10% fetal calf serum to achieve a single cell suspension. The solution was subsequently passed through a cell strainer (Falcon) to remove any debris and the cells were collected into a pellet by centrifugation.

Cell preparation and intracerebral grafting. Fibroblasts were removed from tissue culture plates with 0.05% trypsin and 1 mM EDTA in phosphate-buffered saline (PBS) and suspended in PBS. The fibroblasts were then collected by centrifugation and washed twice in PBS. Mixtures of chromaffin cells and fibroblasts were prepared by combining the cells at a ratio of 1:3 (chromaffin:fibroblasts) in grafting PBS (Dulbecco's PBS, Grand Island, New York supplemented with 2% rat serum) and then diluting the mixture to a final concentration of 100,000 cells/μl (i.e., 100,000 chromaffin cells and 300,000 fibroblasts in 4 μl of PBS). Grafting preparations containing either chromaffin cells or fibroblasts alone were also suspended in grafting PBS at a density of 100,000 cells/μl.

Rats were anesthetized as described previously and placed into a stereotaxic frame for the grafting procedure. The skull was reexposed and a hole was drilled over the dopamine-denervated striatum (coordinates AP +0.7 mm, ML 2.0 mm). A volume of 2 μl of either chromaffin cells with NGF-producing fibroblasts (N = 11), chromaffin cells with Bgal-producing fibroblasts (N = 8), chromaffin cells alone (N = 4), NGF-producing fibroblasts alone (N = 4), or Bgal-producing fibroblasts alone (N = 2) was then injected at a depth of 4.5 mm, the syringe was raised 1 mm and another 2 μl was injected (injection speed 1 μl/2 min). In some animals (N = 12), the chromaffin cell and fibroblast cografts were placed bilaterally into the denervated and intact striata (same coordinates as above) to assess whether chromaffin cell survival was influenced by the denervation.

Immunohistochemical staining. The 25 rats examined at the light microscopic level were sacrificed 8 weeks postgrafting by transcardial perfusion with saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were postfixed overnight at 4°C and transferred to 30% phosphate-buffered sucrose for 3 d at 4°C. Coronal sections (30 μm) were cut on a freezing microtome and collected in 0.1 M Tris-buffered saline (TBS; pH 7.4). Every fourth section through the striatum was stained for Nissl substance using aqueous 0.5% thionin. A second series of every fourth section was immunostained for tyrosine hydroxylase (TH). Selected sections containing grafts were stained for SCG10 (a marker for sympathetic neurons), microtubule associated protein −2 (MAP2), neurofilament 200 kDa (NF-200) and low affinity NGF receptor. Sections processed for immunolabeling were initially treated with a solution of 0.6% aqueous hydrogen peroxide for 30 min, rinsed with TBS, and incubated in a solution containing TBS, 1% horse serum, and 0.25% Triton-X for 1 hr. All antibodies were diluted in this solution and all work was done at room temperature, unless otherwise noted. Sections were incubated overnight with one of the following monoclonal antibodies: mouse anti-TH (Boehringer-Mannheim, 1:200), mouse anti-MAP2 (Sigma, 1:500), mouse anti-NF-200 (Boehringer-Mannheim, 1:24), or mouse anti-NGF receptor (from Chandler et al., 1984; 1:100). The following day, sections were incubated for 1 hr in a solution containing biotinylated horse anti-mouse IgG (Vector Laboratories, 1:150) followed by a second 1 hr incubation in an avidin–biotin solution (Vectastain ABC Elite kit). Both incubations were preceded by thorough rinses in TBS. The reaction product was visualized with 0.03% diaminobenzidine tetrahydrochloride containing 0.01% hydrogen peroxide and 0.02% nickel chloride. The procedure for the polyclonal antibodies, rabbit anti-SCG10 (from David Anderson, 1:500) and rabbit anti-TH (from I. Nagatsu, 1:7500), followed the above protocol except that goat serum was used in place of horse serum and biotinylated goat anti-rabbit (Vector Laboratories, 1:225), rather than horse anti-mouse, was used as the secondary antibody. Also, the rabbit anti-TH was incubated for 2 d at 4°C. The specificity of the each immunoreaction was demonstrated by lack of staining when the primary antibody was omitted from the reaction procedure.

Electron microscopy. The four rats prepared for electron microscopy at 8 weeks postgrafting were perfused with saline followed by a mixture of 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer. After perfusion, the brains were removed and postfixed overnight before being embedded in agar. Horizontal sections (50 μm) cut on a vibratome were examined to locate the graft. Sections containing grafts were osmicated for 1 hr in 1% aqueous OsO_4, dehydrated in an ethanol series, infiltrated with Araldite resins, placed between two acetate sheets, and polymerized for 48 hr at 60°C. After polymerization, one acetate sheet was removed, the flat, embedded tissue photographed, and then

reembedded by inverting a resin filled BEEM capsule over the region containing the graft. After further polymerization, the capsule was removed, trimmed down to the graft region, and sectioned on a Reichert Ultracut E ultramicrotome. Thin sections (70 nm) were collected on 300 mesh copper grids, stained with uranyl acetate and lead citrate, and examined in a Phillips CM 10 electron microscope.

To classify ultrastructural characteristics, grid squares containing graft tissue were photographed sequentially across the graft. All grafted chromaffin cells contained in these fields were counted and classified according to the phenotypic characteristics described in the Results section. Vesicle profile diameter was then determined within three to five cells of each phenotypic classification. All vesicles within these cells were measured from photographic prints using a calibrated ruler with results expressed as mean ± SEM. Only cells clearly within graft boundaries were examined, except for the intentional observation of a host striatal neuron within the host parenchyma. Previous ultrastructural observations of fibroblast grafts have demonstrated that host neuronal cell bodies do not migrate into fibroblast grafts (Kawaja and Gage, 1991). Accordingly, neuronal profiles observed within grafts were graft-derived.

Determination of number and size of TH immunoreactive cells. Using the unbiased optical dissector method (Sterio, 1984) the number of TH-immunoreactive cells within chromaffin cell containing grafts was determined in eight of the rats receiving bilateral implants. Regions of the graft were examined on a video monitor connected to an Olympus Vanox AHS using high numerical aperture objectives (40× or 60×). An unbiased counting frame was printed onto an acetate sheet and placed on the monitor screen. Focusing depth (z-travel) was calibrated using a electronic microcator (Heidenhain model MT12) attached to the microscope stage. The graft was positioned so that the counting frame overlay the dorsal part of the graft. The number of cells in the frame were counted according to the rules for use of the optical dissector (Sterio, 1984). Briefly, the counting frame consists of a known area which was calibrated using a stage micrometer. By focusing into the tissue a known distance (using the microcator) a known miniature volume of the graft is sampled. To be unbiased when counting cells in this miniature volume, it was necessary to have exclusion planes and inclusion planes. Cells contacting the left or bottom edge of the sampling frame on the acetate sheet were not counted. Cell contact with the opposite edges was allowed, similar to counting red blood cells using a hemocytometer. The same principle applied to the optical plane in the z-axis. If a cell was in focus at the first optical depth, but not at the last optical depth, it was counted, the last optical focal plane being the excluded plane. Cells meeting these rules were counted, yielding a number per unit volume or numerical density value (N_v) for the sampled region. The entire graft was sampled by moving the sampling frame on a diagonal and counting from every third position, returning horizontally to the left side of the graft when the right side was reached. Graft boundaries were detected in immunostained material using Nomarski optics. This was done for every fourth section through the graft. The average cellular density for the entire sampled region of the graft was then calculated using the formula $N_v = \Sigma Q/[(A)(H)(\#\text{ of fields sampled})]$, where ΣQ was the number of cells counted, A was the area of the sampling frame, and H was the depth of optical sampling.

Graft volume was then determined using the method of Cavalieri (Michel and Cruz-Orive, 1988). A point counting grid printed on an acetate sheet was placed over the video monitor upon which the whole graft was displayed by using a low power objective (4×). The area around each point was calibrated using a stage micrometer. By knowing the distance between sections and multiplying that by the area per point, each point served as a volume probe. The total volume of the graft (V_{ref}) could then be determined by counting the number of points overlying the graft in semiserial sections through the graft by the formula $V_{ref} = (\Sigma P)(A_p)(t)$ where ΣP was the number of points, A_p was the area associated with each point, and t was the distance between sections. The total number of cells (N_{abs}) in the graft was then determined using the formula $N_{abs} = (N_v)(V_{ref})$.

The maximal diameter of TH immunoreactive cells within the grafts was determined by placing concentric circles of a calibrated diameter over cells displayed on a video monitor (Weibel, 1980). Cells were included only if their nucleus was visible and were measured as a best fit to the appropriate concentric circle.

Statistical analyses. The testing for significant difference of mean value of surviving chromaffin cell number between groups was analyzed by analysis of variance, with a Fisher PLSD post hoc test for determination of significant differences between groups. Differences between means of chromaffin cell size were analyzed by Student's t test (two-tailed). Data are presented as mean ± SEM.

Results

Identification of grafted cells

Sections were stained 8 weeks postgrafting for TH-immunoreactivity (TH-IR) and Nissl substance to localize chromaffin cells, chromaffin-derived neurons and fibroblasts within the grafts. Fibroblasts were observed within all of the fibroblast grafts and cografts, and exhibited characteristic spindle-shaped nuclei in Nissl-stained sections. In the cografts, the fibroblasts were typically located throughout the grafts, while the chromaffin cells and chromaffin-derived neurons were clustered in the central region (Fig. 1*A,B*). Chromaffin cells, identified with TH labeling, were found within all of the chromaffin-containing grafts (Fig. 1*D–F*).

Transdifferentiation of chromaffin cells induced by NGF

In cases in which the chromaffin cells were grafted alone or in combination with control (Bgal) fibroblasts, the TH-IR was localized solely to cell somata (Fig. 1*E,F*, 2*B*). In contrast, chromaffin cells cografted with NGF fibroblasts showed TH-IR both in cell somata and associated neurites which filled the graft with TH-IR (Fig. 1*D*, 2*A*). These neurites were not thick processes, as reported in rats for chromaffin cells exposed for 2 weeks to NGF by genetically modified astrocytes (Cunningham et al., 1991). Instead, the processes surrounding the chromaffin cell somata were very fine, requiring ultrastructural examination for individual resolution (see below). Although there was extensive neurite outgrowth observed within the cografts containing chromaffin-derived neurons and NGF-producing fibroblasts, these processes did not extend into the host brain parenchyma but remained confined within the boundaries of the graft (Fig. 2*A*).

Staining of serial sections for NGF receptor-IR revealed a pattern similar to that seen for TH-IR. Specifically, the cografts of chromaffin cells and NGF-producing fibroblasts were filled with NGF receptor-IR (compare Fig. 3*A* to 2*A*). Significantly less NGF receptor-IR was observed in grafts of NGF fibroblasts alone (Fig. 3*C*), indicating that the majority of the immunoreactivity observed in the chromaffin cell with NGF-producing fibroblast cografts reflected the process outgrowth from the chromaffin cells rather than host-derived cholinergic fiber ingrowth into the grafts. NGF receptor-IR was sparse in chromaffin cell with Bgal fibroblast cografts (Fig. 3*B*) and in Bgal fibroblast grafts (Fig. 3*D*). It is possible that host cholinergic fiber ingrowth, above that observed in the NGF control grafts, is stimulated by the transdifferentiated chromaffin cells. This is, however, considered unlikely. While transdifferentiated chromaffin cells may be producing trophic factors, the most potent attractant known for host cholinergic neurons is NGF. Yet few host cholinergic fibers were attracted to the NGF-producing fibroblast control graft. Further, NF immunoreactive fibers were not observed passing from the host parenchyma to the graft.

NGF-producing fibroblasts were found to induce a marked increase in chromaffin cell diameter compared to the diameters of chromaffin cells grafted alone or with control fibroblasts (Fig. 1*D* vs 1*E,F*). Chromaffin-derived neuron somata within the NGF-cografts averaged 29.1 ± 1.0 μm (n = 345), which was threefold larger than the chromaffin cell diameter (10.5 ± 0.2 μm; n = 462) observed within the control cografts ($p < 0.001$).

Further assessment of the phenotype of chromaffin cells co-

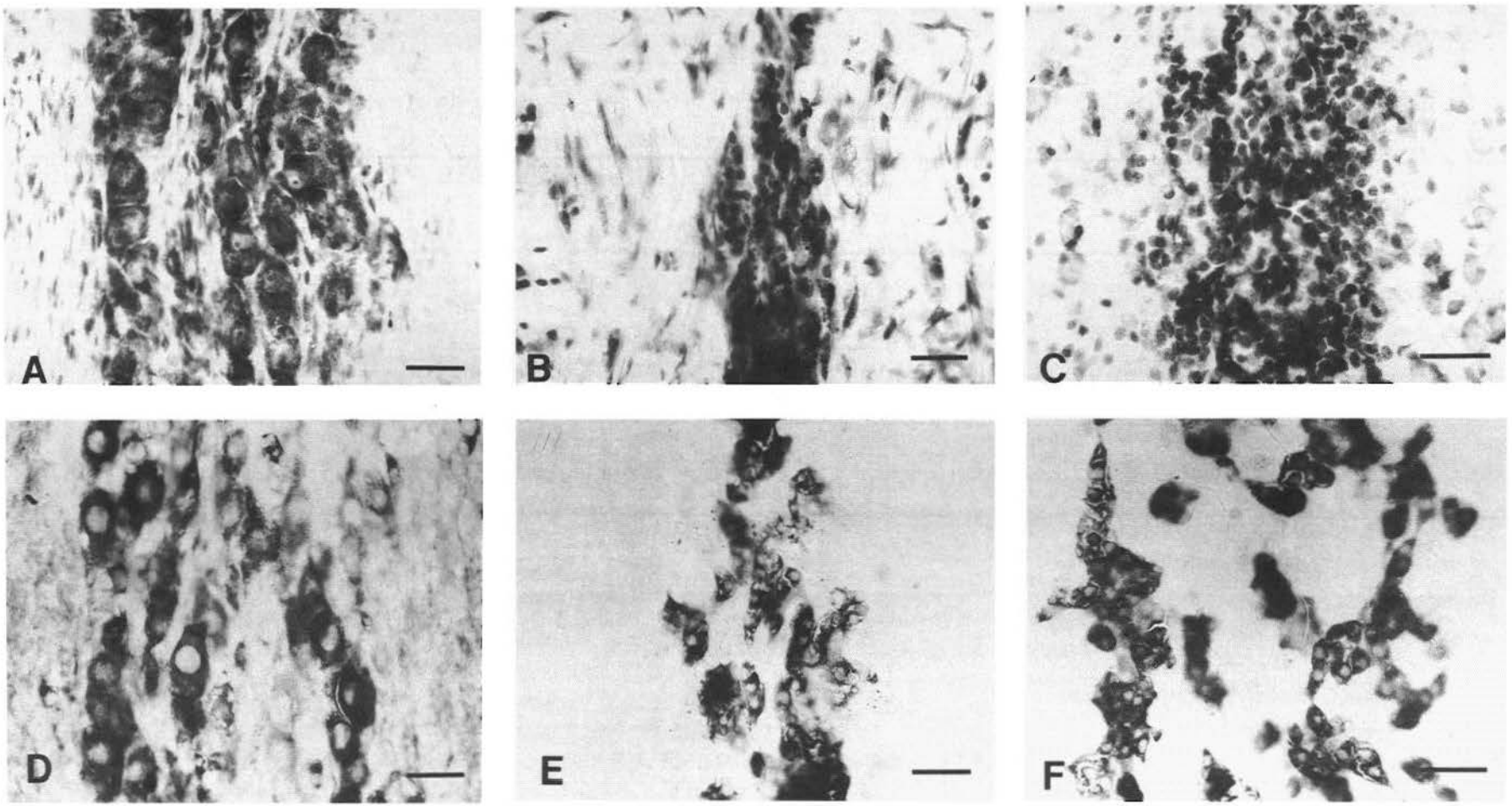


Figure 1. Morphological characteristics of the grafted cells. The sections are stained for Nissl substance (*A–C*) or TH (*D–F*). The grafts contain chromaffin cells plus NGF-producing fibroblasts (*A, D*), chromaffin cells plus control fibroblasts (*B, E*), or chromaffin cells alone (*C, F*). NGF enhances both the survival and the size of TH-immunoreactive perikarya of chromaffin cells (*D* versus *E* or *F*). A significant, but reduced, number of chromaffin cells also survive in the presence of control fibroblasts (*E*) or when chromaffin cells are grafted alone (*F*). Scale bar, 30 μm.

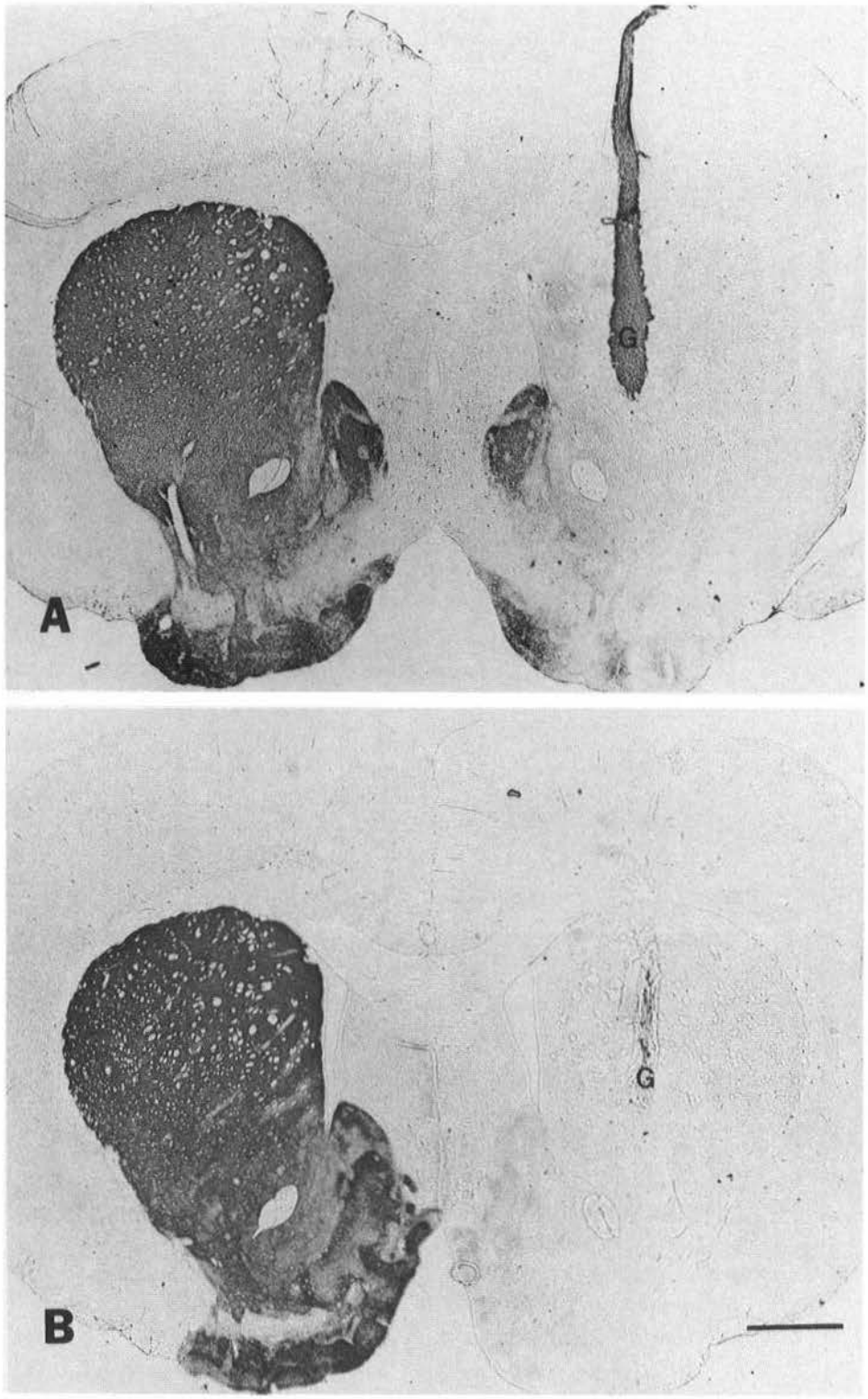


Figure 2. Sections through the striatum of rats that received unilateral 6-OHDA lesions depicting grafts (*G*) of chromaffin cells cografted with NGF-producing fibroblasts (*A*), or chromaffin cells with control fibroblasts (*B*). The sections were stained with mouse anti-TH antibody to illustrate both the extent of the lesions of the nigrostriatal pathway and the locations of chromaffin cell cografts. In (*A*), TH-immunoreactivity fills the entire volume of the graft. In (*B*), surviving TH-immunoreactive chromaffin cells are visible in the central region of the graft. Scale bar, 1 mm.

grafted with the NGF-producing fibroblasts was pursued by staining sections with the neuron-specific markers MAP2 (Olmsted, 1986), SCG10, and NF-200. SCG10 is expressed in the perinuclear cytoplasm, axons, and growth cones of cultured neonatal sympathetic neurons (Stein, 1988a,b). In the NGF cografts, MAP2- and SCG10-IR were observed in cell somata and some processes which were TH-immunoreactive in serial sections (Fig. 4*A* and 4*C*, respectively). Fibers present within the chromaffin/NGF cografts were strongly immunoreactive for NF-200 (Fig. 5*A*) and displayed a pattern of labeling that was similar to that seen for TH-IR (compare to Fig. 1*D*), and for NGF-receptor-IR (compare to Fig. 3*A*). Limited labeling for NF-200 was observed within grafts containing NGF fibroblasts alone (Fig. 5*C*), reflecting the pattern of NGF-receptor-IR that was evident in

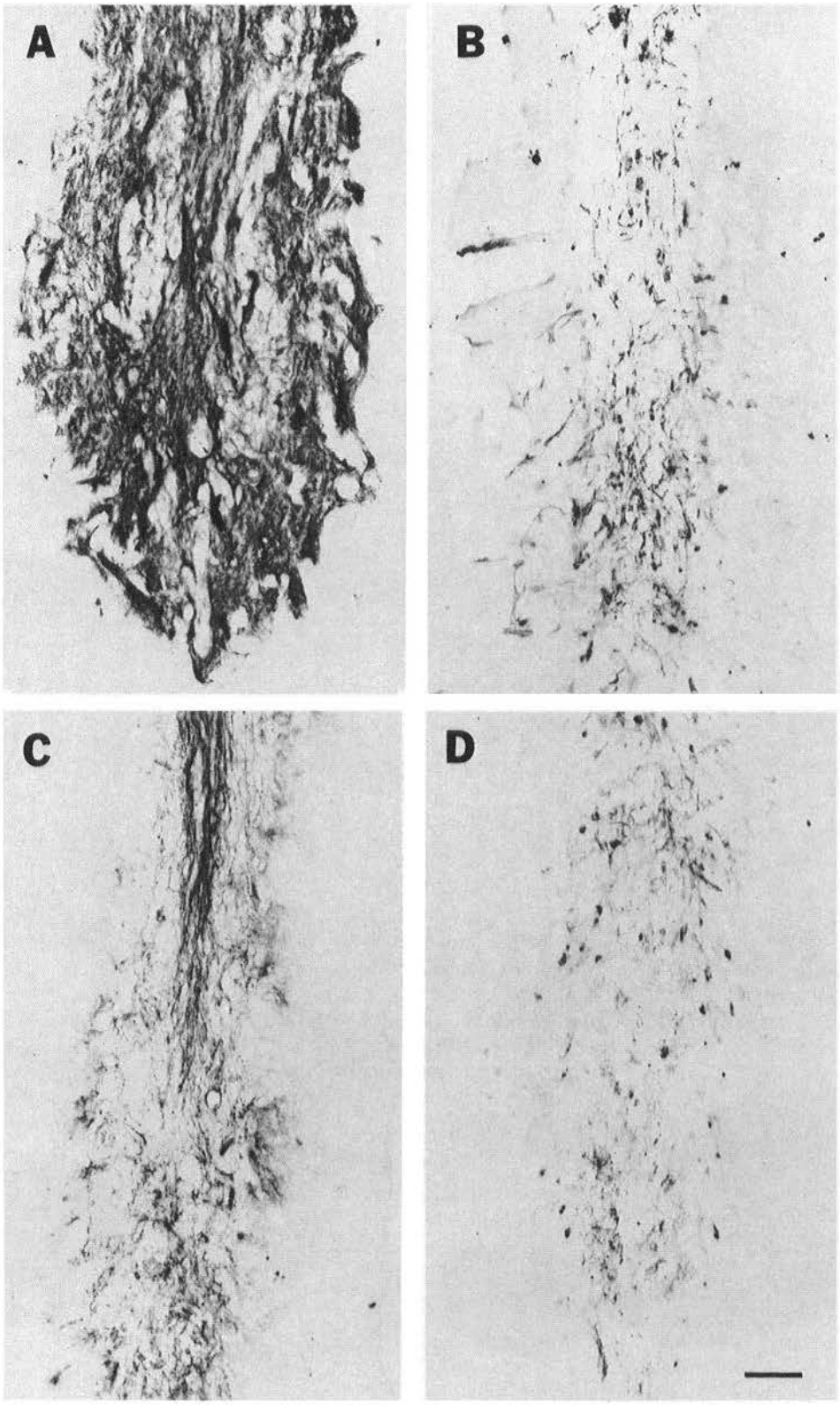


Figure 3. Sections of grafts stained for NGF receptor. Cografts containing chromaffin cells plus NGF-producing fibroblasts are filled with immunostained profiles (*A*), whereas grafts containing chromaffin cells plus control fibroblasts are virtually free of NGF receptor-immunoreactivity (*B*). Some NGF-receptor positive fibers are observed within the NGF-producing fibroblasts-alone graft (*C*) while control fibroblasts-alone graft lacks this immunoreactivity (*D*). Scale bar, 70 μm.

these grafts (see Fig. 3*C*). None of the neuron-specific markers were observed in grafts containing chromaffin cells cografted with Bgal fibroblasts (Figs. 4*B,D*; 5*B*).

Chromaffin cell survival. Assessment of chromaffin cell number within grafts placed in the intact and denervated striata revealed no significant difference between the two sides in either cell number or morphology (Table 1). Dopaminergic denervation of the striatum therefore did not appear to result in the production of factors that affected the survival and differentiation of the grafted chromaffin cells. In contrast, there was a marked influence of NGF on the chromaffin cells *in vivo*. The number of chromaffin cells or chromaffin-derived sympathetic

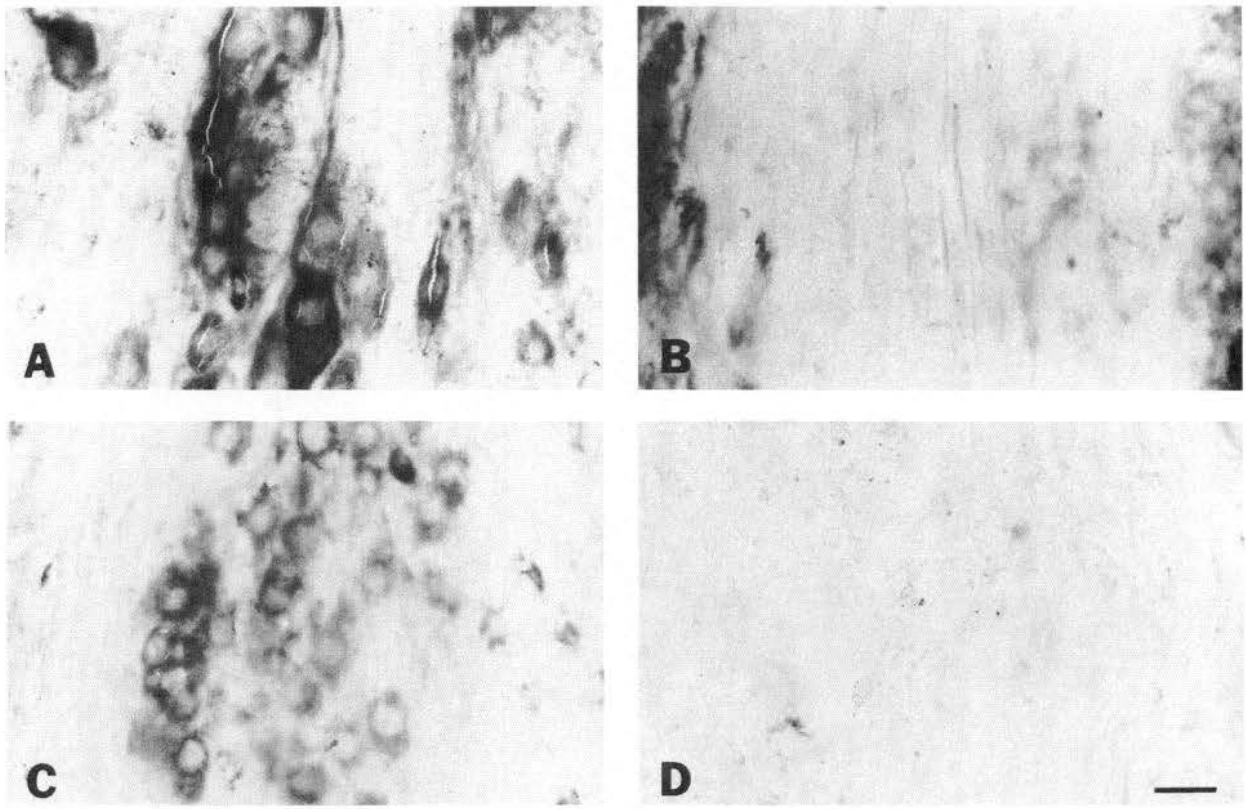


Figure 4. Phenotypic changes in chromaffin cells cografted with NGF-producing fibroblasts are demonstrated with the neuronal-specific markers MAP2 (*A, B*) and SCG10 (*C, D*). Grafts containing chromaffin cells plus NGF-producing fibroblasts (*A, C*) strongly express the markers while chromaffin cells plus control fibroblasts lack staining (*B, D*). Scale bar, 30 μm.

neurons increased from 17% of injected cells in the control cografts to 43% in the presence of the NGF-producing fibroblasts (Table 1). The increased number of chromaffin cells and chromaffin-derived neurons apparent in the NGF cografts may reflect enhanced survival or cell proliferation postgrafting, since NGF has been found to induce mitosis in these cells (Lillien and Claude, 1985). Notably, control fibroblasts also enhanced chromaffin cell number, since only 9% of implanted chromaffin cells were observed in the absence of fibroblasts (Table 1).

Electron microscopic observations. Of the four rats examined ultrastructurally, all had received bilateral cografts of chromaffin cells and fibroblasts, two rats receiving NGF-producing fibroblast cografts bilaterally and two receiving control fibroblast cografts bilaterally. There was no difference observed between the denerved and intact striata. Results described were obtained by examining all grafts within the rats studied ultrastructurally, with figures being representative of the observations made.

Ultrastructural examination revealed dense collagen and vas-

Table 1. Number of surviving chromaffin cells

	6-OHDA–lesioned striatum	Intact striatum	Intact and lesioned striatum combined	% Injected chromaffin cells surviving
Chromaffin cells[a] cografted with NGF-producing fibroblasts[b]	46,259 ± 18,967 (n = 4)	38,792 ± 5569 (n = 4)	42,526 ± 9259 (n = 8)	43%*
Chromaffin cells[a] cografted with control fibroblasts[b]	14,555 ± 7959 (n = 4)	19,305 ± 4401 (n = 4)	16,930 ± 4305 (n = 8)	17%
Chromaffin cells[c] grafted alone	35,178 ± 10,677 (n = 4)	Not done	35,178 ± 10,677 (n = 4)	9%

TH-IR chromaffin cells were counted 8 weeks after grafting into 6-OHDA–lesioned or intact striatum, with control or NGF-producing fibroblasts, or into 6-OHDA–lesioned striatum with no accompanying fibroblasts. Data are presented means ± SEM (cells/graft). The mean value of surviving chromaffin cells cografted with NGF-producing fibroblasts was significantly higher than chromaffin cells cografted with control fibroblasts or grafted alone (*, $p < 0.05$). There was no significant difference in chromaffin cell survival in either type of cografts in the intact versus lesioned striatum.

[a] n = 100,000.

[b] n = 300,000.

[c] n = 400,000.

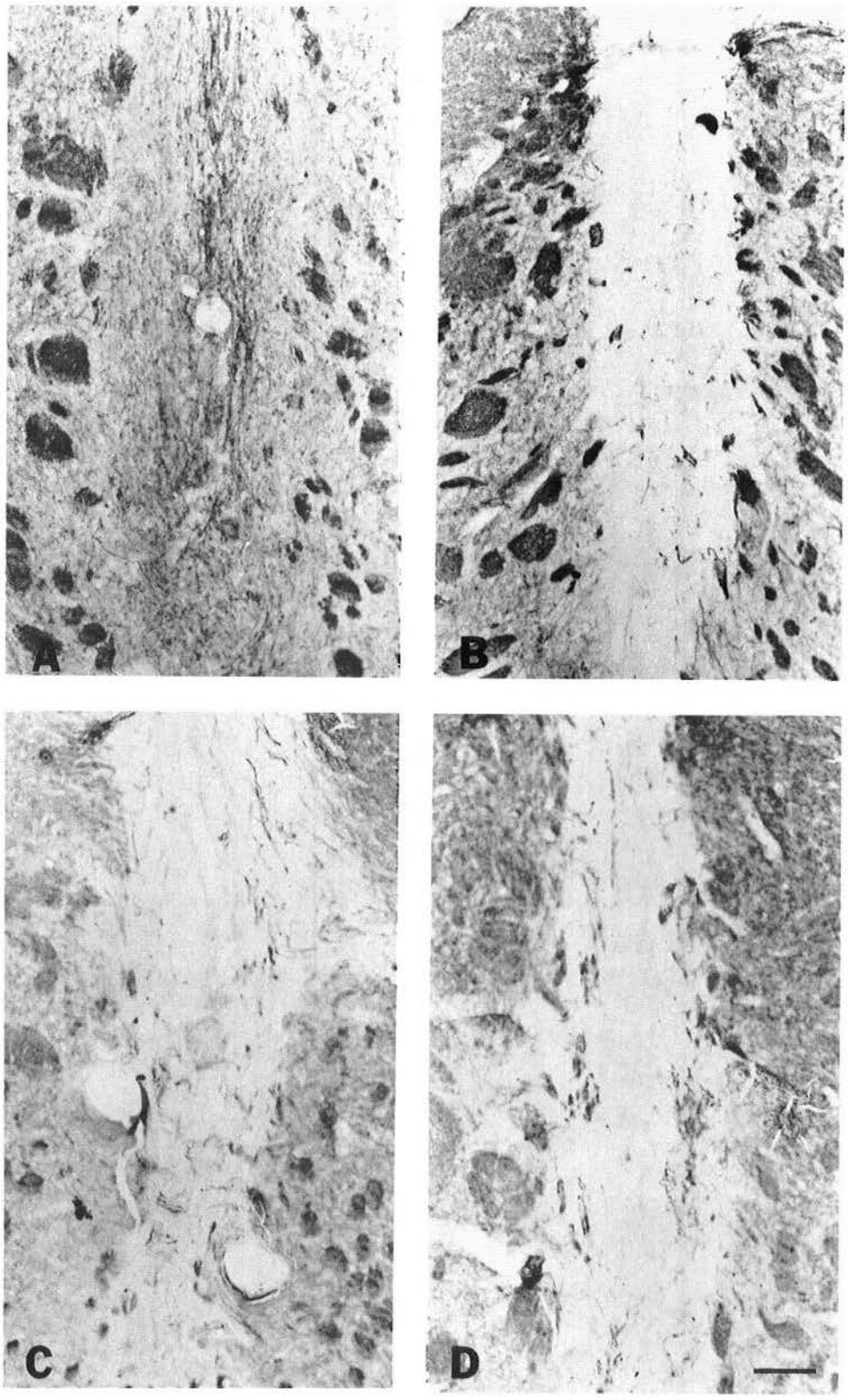


Figure 5. NF-200 staining of grafts reveals profuse immunoreactivity within the graft containing chromaffin cells plus NGF-producing fibroblasts (*A*) while limited immunoreactivity is observed within the NGF-producing fibroblasts-alone graft (*C*). In contrast, cografts containing chromaffin cells plus control fibroblasts (*B*) and grafts of control fibroblasts-alone (*D*) lack NF-200-immunoreactivity. Scale bar, 70 μm.

cularization throughout both types of grafts. While fibroblast morphology was indistinguishable between grafting conditions, chromaffin cell populations could be distinguished between conditions based on the content of the perikaryal cytoplasm. In cografts of chromaffin cells with NGF-producing fibroblasts, 80% of the chromaffin cells (33 of 41 observed) exhibited many ultrastructural features characteristic of neurons, and so were considered to be transdifferentiated. These cells did not possess a basal lamina and contained a euchromatic nucleus, numerous polysomal ribosomes (polysomes), neurofilaments, and small clear vesicles, with a diameter of 76 $\pm$ 2 nm (n = 57) (Fig. 6*A*). This is similar to the neurotransmitter vesicle diameter of 50

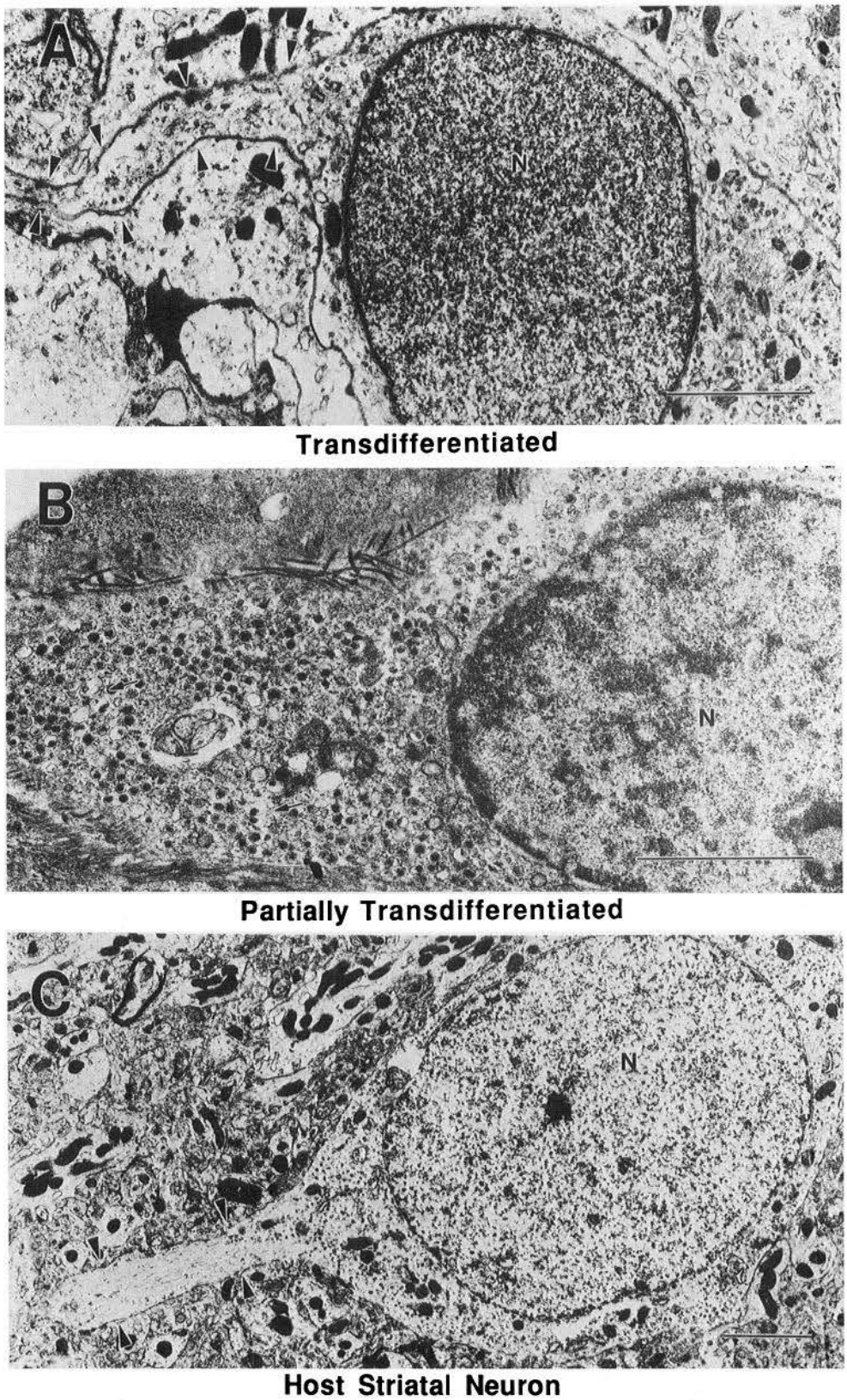


Figure 6. Ultrastructure of chromaffin cells cografted with NGF-producing fibroblasts. *A,* The majority of chromaffin cells exposed to NGF transdifferentiated to demonstrate a number of neuronal characteristics including a euchromatic nucleus (*N*), abundant polysomal ribosomes and neurofilaments, some small clear vesicles, neurites (bracketed by *arrowheads*), and an absence of dense cored vesicles. *B,* Some chromaffin cells exposed to NGF were only partially transdifferentiated. These cells contain a heterochromatic nucleus (*N*) and a perikaryal cytoplasm filled with dense cored vesicles. Most of these vesicles are small, but some larger vesicles (*arrows*) such as those in nontransdifferentiated chromaffin cells (see Fig. 7*A*) are present. *C,* Host striatal neuron for comparison with the transdifferentiated chromaffin cells. Note the euchromatic nucleus (*N*), absence of dense cored vesicles, presence of polysomal ribosomes and neurofilaments within the perikaryal cytoplasm and a neurite (bracketed by arrowheads) similar to that observed in transdifferentiated chromaffin cells (*A*). Scale bars, 2 μm.

nm in sympathetic neurons (Anderson, 1989) and 70 nm in central neurons (Peters et al, 1991). Fine caliber processes were occasionally seen to emerge from these transdifferentiated chromaffin cells (Fig. 6*A*). The other 20% of the chromaffin cells within the NGF-producing fibroblast cografts were partially transdifferentiated as indicated by a heterochromatic nucleus, an abundance of compact dense cored vesicles (diameter = 103 ± 2 nm, $n = 97$), as well as small clear vesicles (diameter = 73

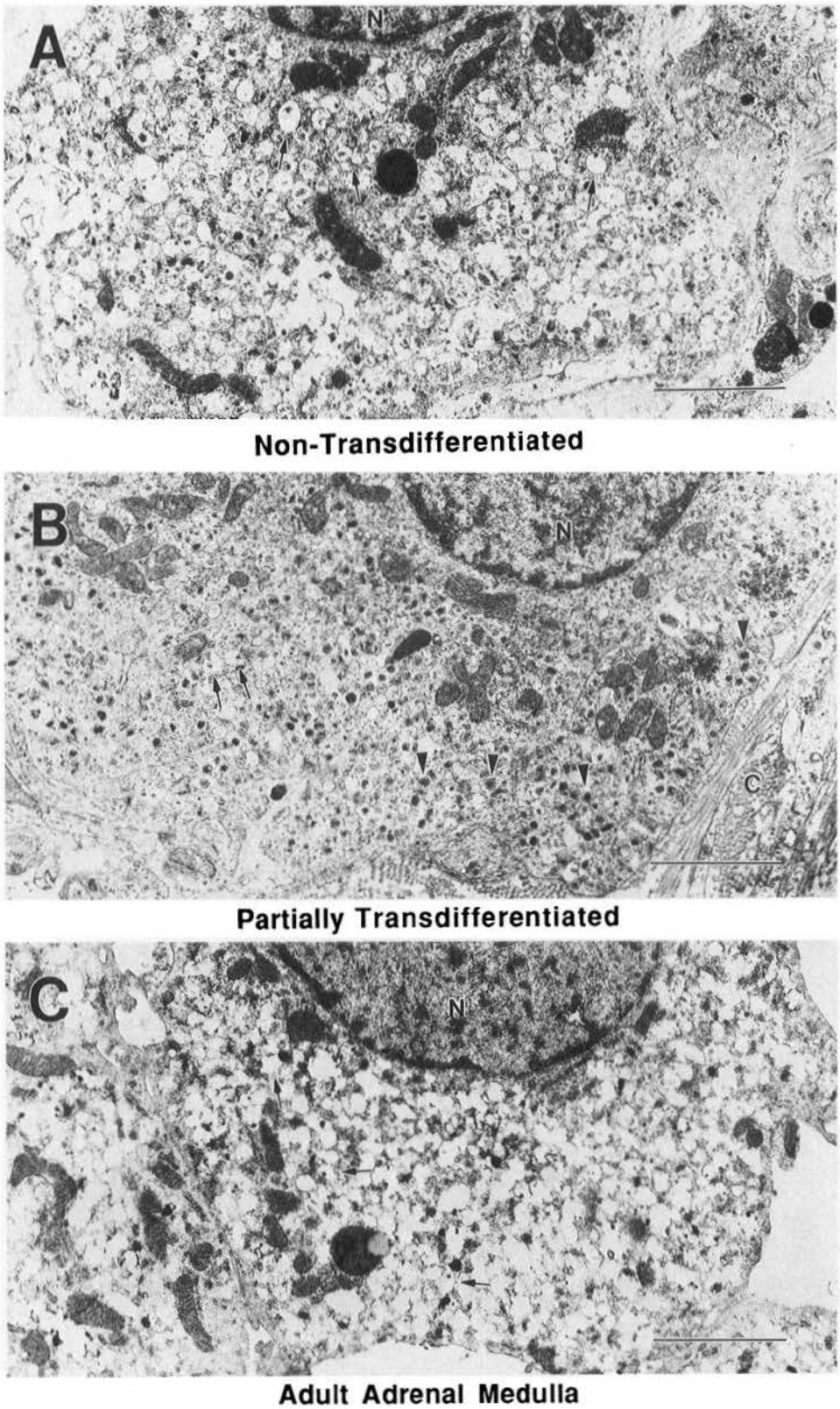


Figure 7. Ultrastructure of chromaffin cells cografted with control fibroblasts. *A*, More than half of the chromaffin cells cografted with control fibroblasts retained a nontransdifferentiated phenotype as characterized by the dominance of large dense cored vesicles (*arrows*) and a heterochromatic nucleus (*N*). *B*, Many of the chromaffin cells cografted with control fibroblasts were partially transdifferentiated, retaining a heterochromatic nucleus (*N*), but also containing a mixed population of large (*arrows*) and small (*arrowheads*) dense cored vesicles. Collagen (*C*) produced by the cografted fibroblasts is present between cells. *C*, Chromaffin cells from the adult adrenal medulla for comparison with nontransdifferentiated chromaffin cells. This *in situ* adrenal chromaffin cell has a heterochromatic nucleus (*N*) with a cytoplasm dominated by large vesicles (mean profile diameter 252 ± 4 nm, $N = 108$) possessing dense cores (*arrows*) similar to that seen in a nontransdifferentiated grafted chromaffin cell (*A*). Scale bars, 2 μm.

± 3 nm, $n = 26$) (Fig. 6*B*). These chromaffin cells also contained polysomes and neurofilaments. For morphological comparison, a host neuron from the adjacent striatum is shown (Fig. 6*C*).

The perikaryal cytoplasm of the majority (55%, 17 of 31 observed) of chromaffin cells cografted with control fibroblasts was dominated by numerous dilated vesicles (diameter = 302 ± 6 nm, $n = 114$), many of which contained dense cores (Fig. 7*A*). It is probable that the number of vesicles with dense cores observed was underestimated due to the low probability of a single thin section through a large vesicle transecting the dense

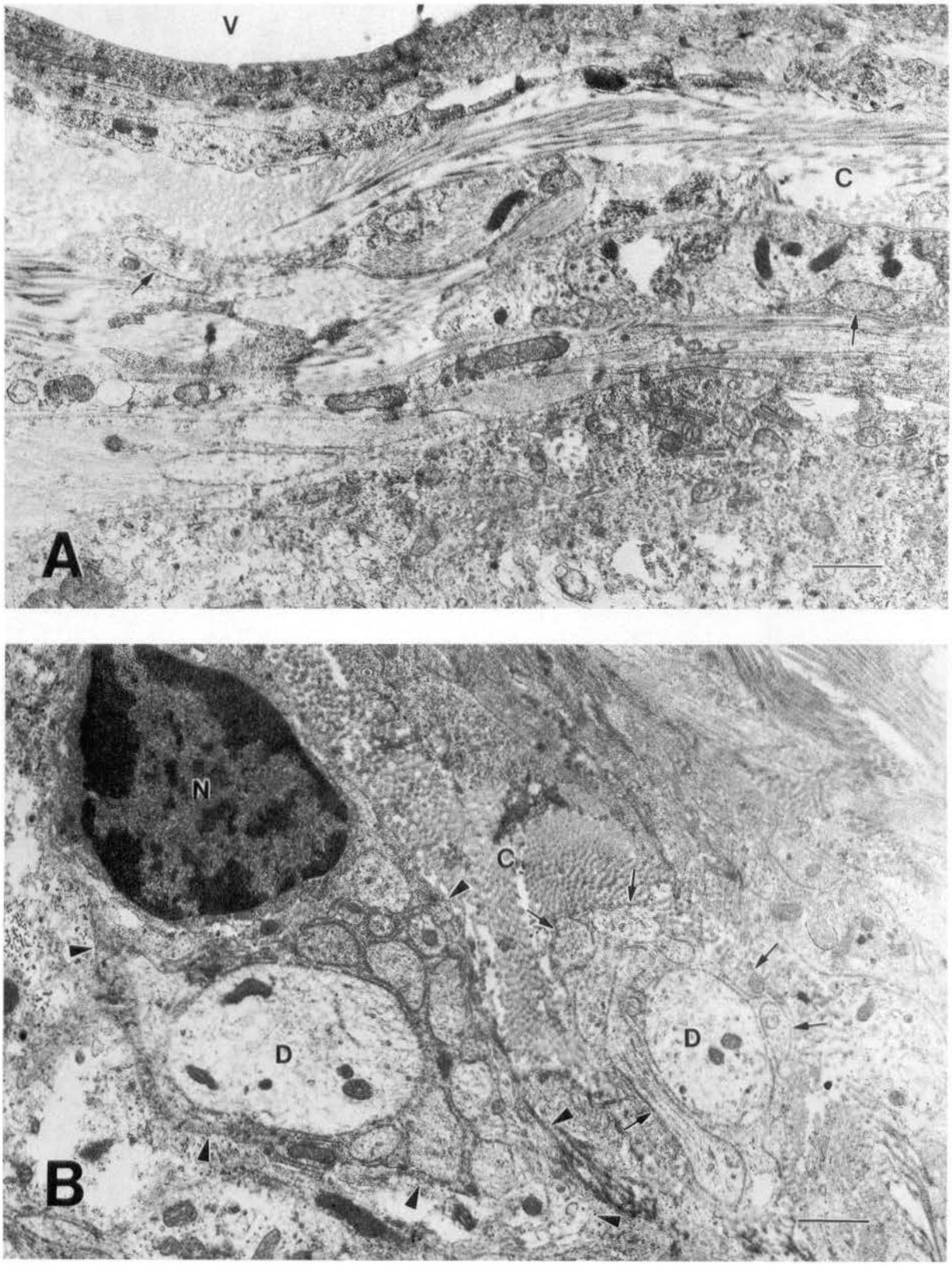
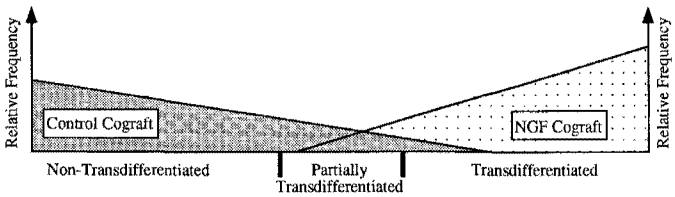


Figure 8. Neuronal profiles in cografts of chromaffin cells and NGF-producing or control fibroblasts. *A,* Within cografts of chromaffin cells and control fibroblasts, abundant collagen (*C*) and fibroblast processes are seen, while very few neuronal processes (*arrows*) are observed. *V,* Small blood vessel. *B,* Cografts of NGF-producing fibroblasts and chromaffin cells contain abundant neuronal processes (*arrows*) bundled together in fascicles (*arrowheads*). Processes were identified as dendrites (*D*) or axons (*arrows*) which contained numerous small clear vesicles. *N,* Nucleus; *C,* collagen. Scale bars, 1 μm.

core. Other prominent organelles were mitochondria, rough endoplasmic reticulum, and Golgi. These chromaffin cells possessed a basal lamina around the plasmalemma, a heterochromatic nucleus and few, if any, intermediate filaments (Fig. 7*A*). These characteristics are similar to those observed in chromaffin cells *in situ* (Fig. 7*C*) indicating that these grafted chromaffin cells were nontransdifferentiated. Approximately one-third (29%, 9 of 31 observed) of chromaffin cells cografted with control fibroblasts showed a partially transdifferentiated phenotype with a heterochromatic nucleus and perikaryal cytoplasm dominated by a mixed population of vesicles, which varied in size from 77 to 385 nm (mean diameter = 158 ± 6, $n = 113$) (Fig. 7*B*). These cells possessed a basal lamina and contained rosettes of polysomes and occasional neurofilaments, differing from the partially transdifferentiated population described above for NGF fibroblast cografts by the presence of large dense core vesicles. A small population of chromaffin cells cografted with control fibroblasts possessed a transdifferentiated phenotype (16%, 5 of 31 observed). The soma of these cells appeared similar to that described for transdifferentiated chromaffin cells in NGF cografts described above. The relative frequency of observation of the three chromaffin derived cell types seen in the two types of grafts is summarized in Figure 9.

In agreement with the light microscopic examination, cografts of chromaffin cells with NGF-producing fibroblasts contained many neuronal processes, usually in fascicles (Fig. 8*B*). These processes were arranged into morphologically distinct axons of small caliber containing mitochondria, parallel arrays of microtubules, accumulated small, clear vesicles and some morphologically distinct dendrites of large caliber containing floccular cytoplasm with mitochondria, neurofilaments, and microtubules. In contrast, neuronal processes were only occasionally observed in the grafts of chromaffin cells and control fibroblasts (Fig. 8*A*). These processes contained microtubules with few vesicles and were most commonly found singly, not in fasicules. No dense cored vesicles were observed in the neuritic profiles observed under either grafting condition. Synaptic membrane specialization was rarely observed in the NGF-producing cografts and not at all in the control fibroblast cografts.



Characteristic	Non-Trans-differentiated	Partially Trans-differentiated	Trans-differentiated
heterochromatic nucleus	+	+	-
euchromatic nucleus	-	-	+
large dense cored vesicles	++	+	-
small dense cored vesicles	-	+	-
small clear vesicles	-	+	+
neurofilaments	-	+	++
polysomal ribosomes	-	+	++
basal lamina	+	+	-

Figure 9. Schematic indicating the relative frequency of grafted chromaffin cells exhibiting different ultrastructural characteristics. Most chromaffin cells cografted with control fibroblasts (*dark stipple*) were nontransdifferentiated, while a small number were partially or completely transdifferentiated toward a sympathetic neuron phenotype. Most chromaffin cells cografted with NGF-producing fibroblasts (*light stipple*) were transdifferentiated, expressing many neural characteristics, while a few were only partially transdifferentiated. The characteristics used to assess the degree of transdifferentiation is summarized below. +, Feature present; ++, feature predominant; −, feature absent.

Discussion

We have found that chromaffin cells cografted with NGF-producing fibroblasts can transdifferentiate to express many neuron-specific characteristics *in vivo,* in the adult rat brain. In addition, chromaffin cell numbers within grafts in the striatal parenchyma can be greatly enhanced by cografting with NGF-producing fibroblasts.

Phenotypic plasticity of chromaffin cells grafted in the brain

Chromaffin cells cografted with NGF-producing fibroblasts displayed many properties characteristic of mature sympathetic neurons as assessed by morphological criteria (Burnstock and Costa, 1975; Doupe et al., 1985) and by staining for the neuron-specific immunohistochemical markers. These sympathetic neuron-like cells had profuse TH, NF-200 and NGF receptor-immunoreactive processes. While double labeling was not performed, the degree of similarity between the TH-, NF-200-, and NGF receptor-immunoreactivity in the cografts of chromaffin cells with NGF-producing fibroblasts (Figs. 2*A*, 3*A*, 5*A*) strongly suggests that the same neuritic processes were being stained by all of these antibodies. Neurites from transdifferentiated rat chromaffin cells *in vitro* are similarly positive for TH and neurofilament (Doupe et al., 1985). In addition, the low affinity form of the NGF receptor is expressed on the neuritic processes of transdifferentiated monkey chromaffin cells (Herman et al., 1992). The MAP2 antibody used is selective for neurons, labeling dendritic trees throughout the brain. It does not stain chromaffin cells grafted alone or cografted with control fibroblasts. SCG10 is expressed in sympathetic neurons but not in chromaffin cells (Anderson and Axel, 1985) and it can be induced by NGF during transdifferentiation of chromaffin cells into sympathetic neurons (Stein et al., 1988b). The expression of neuron-specific NF-200, MAP2, and SCG10 in chromaffin cells cografted with NGF-producing fibroblasts, therefore, indicated the neuronal transdifferentiation of these cells.

Ultrastructural examination further revealed the transdifferentiation of chromaffin cells cografted with NGF-producing fibroblasts. Without NGF exposure, most of the chromaffin cells cografted with control fibroblasts resembled chromaffin cells *in situ* with their very large, dense cored vesicles. The average vesicle size observed in the nontransdifferentiated chromaffin cells was similar to that observed in chromaffin cells *in situ.* This endocrine cell morphology is also similar to that observed when chromaffin cells are cultured with glucocorticoids but without NGF (Doupe et al., 1985; Hansen et al., 1989). In contrast, most of the chromaffin cells observed in the cografts with NGF-producing fibroblasts exhibited a neuronal morphology exemplified by their cytoplasmic organelles. The ultrastructural characteristics observed in the chromaffin cells transdifferentiated *in vivo* appeared similar to the characteristics of sympathetic neurons *in vivo* (Peters et al., 1991), and to those reported for chromaffin-derived sympathetic neurons *in vitro* (Doupe et al., 1985).

In addition to the distinct endocrine-like morphology of the nontransdifferentiated chromaffin cells and the neuron-like morphology of transdifferentiated cells, there was an intermediate cell type observed. These chromaffin cells may have been in a transition towards a neuronal phenotype. Similar transi-

tional cells, with properties of both chromaffin cells and neurons, have been reported when chromaffin cells are transdifferentiated *in vitro* (Doupe et al., 1985; Notter et al., 1989). The primary neuronal features of these transitional cells are compact dense cored vesicles and the appearance of polysomes. The presence of some chromaffin cells cografted with control fibroblasts with ultrastructural characteristics of partially or fully transdifferentiated neurons indicates that phenotypic changes were occurring that were not detectable at the light microscopic level. This suggests that a factor capable of stimulating chromaffin cell transdifferentiation was locally available to the chromaffin cells. Of the factors known to induce chromaffin cell transdifferentiation, bFGF and NGF may be the most likely candidates because fibroblasts normally produce NGF at low levels (Yoshida and Gage, 1992), and transient increases in bFGF levels are reported following brain injury (Logan et al., 1991). Even chromaffin cells grafted alone to the striatum may undergo limited transdifferentiation in some cases, as reflected by the development of short neuritic processes on some cells (Freed et al., 1981; Bing et al., 1988, 1990).

Ultrastructural characteristics of neuronal processes within the cografts were consistent with light microscopic observations. Neuritic profiles were seldom encountered within the grafts containing control fibroblasts while cografts of NGF-producing fibroblasts contained numerous neuritic profiles organized into fascicles. The neurites observed were characteristic of mature, differentiated neurons in that they contained microtubule arrays, neurofilaments, and small, clear vesicles. None of the neurite profiles observed in either grafting condition contained dense cored vesicles, in contrast to a previous *in vitro* report where occasional granular vesicles were found in chromaffin derived sympathetic neurons (Doupe et al, 1985). The lack of synapses may be the result of a lack of appropriate target sites, although synapse formation between chromaffin cell-derived neurons has been reported *in vitro* (Doupe et al., 1985).

The TH-IR neurites observed filling the cografts of chromaffin cells with NGF-producing fibroblasts appear to derive from the transdifferentiated chromaffin cells rather than from the host. This was supported by the lack of such immunoreactivity within any of the control grafts, particularly the graft of NGF-producing fibroblasts alone, which suggests that the TH-IR observed in the grafts of chromaffin cells with NGF-producing fibroblasts was not due to ingrowth of peripheral sympathetic axons. Further, the complete removal of dopaminergic input to the striatum by the 6-OHDA treatment (Fig. 2) eliminated the possibility that the TH-IR arose from spared dopaminergic fibers.

The very fine processes extending from the chromaffin cells under the influence of NGF that were observed in the present study were not morphologically similar to those described in previous grafting experiments (e.g., Stromberg et al., 1985; Kordower et al., 1990; Cunningham et al., 1991). Relatively thick TH-IR processes tapering to thinner processes were observed extending from rat chromaffin somata after 2 weeks of exposure to NGF (Stromberg et al., 1985; Cunningham et al., 1991). Similarly, very thick neurites extended from monkey chromaffin cells 3 months after cografting with sural nerve segments (Kordower et. al., 1990). We have observed (unpublished observations) similar thick processes from chromaffin cells *in vivo* after 2 weeks exposure to a combination of basic fibroblast growth factor and NGF. Four weeks of similar treatment, however, resulted in thin processes similar to the type reported here. It is possible that a progressive change in morphology over time represents a developmental process in the formation of the chromaffin cell neurites *in vivo*. Differences in neurite thickness may also be due in part to differences in the extracellular matrix around the chromaffin cells. For chromaffin cells cografted with fibroblasts there is an abundance of collagen in the grafts, which would not be expected for chromaffin cells cografted with astrocytes or sural nerve segments.

The increase in cell body size that was observed for chromaffin cells cografted with NGF-producing fibroblasts further indicates that the transdifferentiated chromaffin cells expressed characteristics of sympathetic neurons. Sympathetic neurons have a much larger soma size than chromaffin cells, 40–50 μm versus 20 μm (Doupe et al., 1985). Taken together, the morphological, histochemical and ultrastructural evidence indicates that the chromaffin cells exposed to NGF *in vivo* were well transdifferentiated and displayed a number of characteristics of mature sympathetic neurons, (e.g., increased soma size, neurite formation, decreased vesicle size, and expression of neuron specific proteins). The cells, however, did not transdifferentiate as completely as reported by *in vitro* studies (Doupe et al., 1985). For example, there was no evidence of synaptic connections from the neurites and, while the vesicle size was decreased, the presence of vesicles in the somata is atypical for sympathetic neurons. This latter observation is similar to the result observed when adult-derived chromaffin cells were transdifferentiated *in vitro* (Doupe et al., 1985).

Enhancement of the survival of grafted chromaffin cells

We observed that chromaffin cell number was significantly enhanced by exposure to NGF, as others have reported (Stromberg et al., 1985; Date et al., 1990; Kordower et al., 1990; Cunningham et al., 1991). Although dopamine-denervated striatum expresses some factor(s) that promote the survival of dopaminergic neurons *in vitro* (Dal Toso et al., 1988; Niijima et al., 1990; Hyman et al., 1991), there is no evidence in our study that such a factor influenced chromaffin cell number *in vivo*. For studies that estimate the number of chromaffin cells implanted, the percentage of surviving cells can be determined. In the monkey, Kordower and coworkers (Kordower et al., 1990) report 3 month intraparenchymal survival of 1.5% and 12.5% of implanted chromaffin cells without and with sural nerve cografts, respectively. The nerve segments cografted with the chromaffin cells produce a variety of trophic factors that could singularly, or in combination, affect chromaffin cell survival. In addition to NGF (Bandtlow et al., 1987), these factors include, ciliary neurotrophic factor (Manthorpe et al., 1986), acidic fibroblast growth factor (Eckenstein et al., 1991), and brain derived neurotrophic factor (Acheson et al., 1991). Doering (1992) also demonstrated that peripheral nerve is able to provide an environment which permits large numbers of chromaffin cells to survive in the brain for up to 6 months. Although the number of implanted chromaffin cells was not determined by Doering (1992), there was no loss of cells between 2 weeks and 6 months.

Cunningham and coworkers (Cunningham et al., 1991) reported that 2 weeks after grafting, chromaffin cell survival was 1.5%, 0.7%, and 3.8% for chromaffin cells implanted alone, with control astrocytes or with NGF-producing astrocytes, respectively. NGF production rate *in vitro* was determined to be 9.3 pg NGF/10^5cells/hr from the genetically modified astrocytes (Cunningham et al., 1991). This was far less than the rate of 900–1000 pg NGF/10^5cells/hr, as determined by two-site immunoassay for the NGF-producing fibroblasts used in the pres-

ent study (unpublished observations). In addition to a higher rate of NGF production per cell, the ratio of NGF-producing cells to chromaffin cells was higher in the present study compared to Cunningham et al. (1991), 3:1 versus 1:1. The exposure to a higher concentration of NGF may be responsible for the increased number of chromaffin cells observed 8 weeks postgrafting in the present report, as well as for some of the differences in neurite morphology discussed above.

While we did not assay for NGF production by the transfected fibroblasts *in vivo*, the present study and previous work with these cells (Eagle et al., 1992), suggest that NGF is produced for a considerable period of time after grafting. Expression of SCG10 mRNA is reversible and requires the continued presence of NGF in PC12 cells (Stein et al., 1988b) indicating that the chromaffin cells with SGC10-IR in the present study must have been continuously exposed to NGF for the 8 weeks examined. In addition, chromaffin cells converted to a neuronal phenotype by NGF *in vitro* continue to require NGF for survival, even after maturation (Chun and Patterson, 1977a,b). The source of the presumed NGF in the graft was likely to be the NGF gene transfected into the fibroblasts because chromaffin cells grafted alone or with control fibroblasts did not express SCG10 and the other neuronal markers studied.

An unexpected finding in this study was that control fibroblasts enhanced chromaffin cell survival, compared to chromaffin cells grafted alone. This increased survival, possibly due to a low level of NGF production by normal fibroblasts (Yoshida and Gage, 1992), is in contrast to recent findings by Schueler and coworkers (Schueler et al., 1993). Schueler et al. (1993) found that when dissociated bovine chromaffin cells were implanted into cyclosporin A treated rats, that chromaffin cell survival was enhanced by removal of fibroblasts, endothelial cells, and other cell types. Further, when fibroblasts and endothelial cells were recombined with the chromaffin cells from which they were isolated, chromaffin cell survival was reduced. These results indicated that nonchromaffin cell types in the adrenal medulla, particularly fibroblasts and endothelial cells, inhibit chromaffin cell survival following grafting (Schueler et al., 1993). A comparison between the present results, where the addition of control fibroblasts enhanced chromaffin cell number, and those of Schueler and coworkers, where the removal of nonchromaffin cells including fibroblasts enhanced chromaffin cell survival, can not be directly made due to differences between the two model systems. In the present study, autologous skin fibroblasts were added to the mixture of dissociated autologous adrenal medulla cells to be grafted. In contrast, Schueler et al. (1993) were utilizing xenografts in which the adrenal derived fibroblasts and endothelial cells removed or added were from the same species as the chromaffin cells used, but a different species from the host. It is possible that these different methodologies which lead to enhancement of chromaffin cell survival (i.e., addition vs removal of other cell types including fibroblasts) may be due to differences in the species source of the adrenal medulla and/or the tissue source of the fibroblasts used.

Innervation of host brain by grafted chromaffin cells

Although the chromaffin cells exposed to NGF were converted into cells expressing characteristics of sympathetic neurons and survived at least 8 weeks *in vivo*, we found no evidence of neurite outgrowth from the graft into the host striatum. Since the level of endogenous expression of NGF in the striatum of adult rats is extremely low (Korsching et al., 1985), it is likely that a steep, downward gradient of NGF concentration exists between the graft and the host striatum. It is known that neurites from sympathetic neurons do not grow from areas with high NGF levels into areas with low NGF (Campenot, 1982). The innervation of host tissue by chromaffin-derived neurites observed by Stromberg and coworkers (Stromberg et al., 1985) suggests that a very high local concentration of NGF was being released from the infusion pumps used in their study. To enhance outgrowth into the host striatum in the current cograft model, it may be necessary to either induce chromaffin cell transdifferentiation with factors other than NGF, or implant sources of NGF elsewhere in the striatum (Chalmers et al., 1993).

Conclusions

The present study demonstrates that chromaffin cells transdifferentiate *in vivo* under the influence of NGF and express many phenotypic features of sympathetic neurons. In addition, the environment produced by the cografts of chromaffin cells with fibroblasts was able to result in chromaffin cell survival far in excess of that reported previously, with NGF-producing fibroblasts having the greatest effect. Future experiments will be necessary to determine if the chromaffin cell neurites can be induced to leave the graft environment and enter the host parenchyma. Also, it will be important to determine if catecholamines are being released from the transdifferentiated chromaffin cells and if animal behavior is modified in models of striatal dopamine deficiency.

References

Acheson A, Barker PA, Alderson RF, Miller FD, Murphy RA (1991) Detection of brain-derived neurotrophic factor-like activity in fibroblasts and Schwann cells: inhibition by antibodies to NGF. Neuron 7:265–275.

Anderson DJ (1989) Cell "Neoteny." A possible developmental basis for chromaffin cell plasticity. Trends Genet 5:174–178.

Anderson DJ, Axel R (1985) Molecular probes for the development and plasticity of neural crest derivatives. Cell 42:649–662.

Anderson DJ, Axel R (1986) A bipotential neuroendocrine precursor whose choice of cell fate is determined by NGF and glucocorticoid. Cell 47:1079–1090.

Bandtlow CE, Heumann R, Schwab ME, Thoenen H (1987) Cellular localization of nerve growth factor synthesis by *in situ* hybridization. EMBO J 6:891–899.

Bing G, Notter MF, Hansen JT, Kellogg C, Kordower JH, Gash DM (1990) Cografts of adrenal medulla with C6 glioma cells in rats with 6-hydroxydopamine-induced lesions. Neuroscience 34:687–697.

Bing GY, Notter MF, Hansen JT, Gash DM (1988) Comparison of adrenal medullary, carotid body and PC12 cell grafts in 6-OHDA lesioned rats. Brain Res Bull 20:399–406.

Bohn MC, Cupit L, Marciano F, Gash DM (1987) Adrenal medulla grafts enhance recovery of striatal dopaminergic fibers. Science 237: 913–916.

Burnstock G, Costa M (1975) Adrenergic neurons. New York: Wiley.

Campenot RB (1982) Development of sympathetic neurons in compartmentalized cultures. II. Local control of neurite growth by nerve growth factor. Dev Biol 93:1–12.

Chalmers GR, Fisher LJ, Niijima K, Patterson PH, Gage FH (1993) Outgrowth from differentiated chromaffin cells induced by NGF-producing fibroblast grafts. Soc Neurosci Abstr 19:1319.

Chandler CE, Parson LM, Hosang M, Shooter EM (1984) A monoclonal antibody modulates the interaction of nerve growth factor with PC 12 cells. J Biol Chem 259:6882–6889.

Chun LL, Patterson PH (1977a) Role of nerve growth factor in the development of rat sympathetic neurons *in vitro*. I. Survival, growth, differentiation of catecholamine production. J Cell Biol 75:694–704.

Chun LL, Patterson PH (1977b) Role of nerve growth factor in the development of rat sympathetic neurons *in vitro*. II. Developmental studies. J Cell Biol 75:705–711.

Cunningham LA, Hansen JT, Short MP, Bohn MC (1991) The use of

genetically altered astrocytes to provide nerve growth factor to adrenal chromaffin cells grafted into the striatum. Brain Res 561:192–202.

Dal Toso R, Giorgi O, Soranzo C, Kirschner G, Ferrari G, Favaron M, Benvegnu D, Presti D, Vicini S, Toffano G, Azzone GF, Leon A (1988) Development and survival of neurons in dissociated fetal mesencephalic serum-free cell cultures: 1. Effects of cell density and of an adult mammalian striatal-derived neurotrophic factor (SDNF). J Neurosci 8:733–745.

Date I, Felton SY, Felton DL (1990) Cografts of adrenal medulla with peripheral nerve enhance the survivability of transplanted adrenal chromaffin cells and recovery of the host nigrostriatal dopaminergic system in MPTP-treated young adult mice. Brain Res 537:33–39.

Doering LC (1992) Peripheral nerve segments promote consistent long-term survival of adrenal medulla transplants in the brain. Exp Neurol 118:253–260.

Doering LC, Birren SJ, Anderson DJ (1992) Transplants of the sympathoadrenal (MAH-B2) cell line survive and differentiate within the adult peripheral nervous system. Soc Neurosci Abstr 18:783.

Doupe AJ, Landis SC, Patterson PH (1985) Environmental influences in the development of neural crest derivatives: glucocorticoids, growth factors, chromaffin cell plasticity. J Neurosci 5:2119–2142.

Eagle KL, Fisher LJ, Chalmers GR, Gage FH (1992) Functional and morphological characterization of NGF-producing grafts implanted into lesioned rat fimbria-fornix. Soc Neurosci Abstr 18:1121.

Eckenstein FP, Shipley GD, Nishi R (1991) Acidic and basic fibroblast growth factors in the nervous system: distribution and differential alteration of levels after injury of central versus peripheral nerve . J Neurosci 11:412–419.

Fine A (1990) Transplantation of adrenal tissue into the central nervous system. Brain Res Rev 15:121–133.

Freed WJ, Morihisa JM, Spoor E, Hoffer BJ, Olson L, Seiger A, Wyatt RJ (1981) Transplanted adrenal chromaffin cells in rat brain reduce lesion-induced rotational behaviour. Nature 292:351–352.

Freed WJ, Cannon-Spoor HE, Krauthamer E (1986) Intrastriatal adrenal medulla grafts in rats: long term survival and behavioral effects. J Neurosurg 65:664–670.

Freed W, Poltorak M, Becker JB (1990) Intracerebral adrenal medulla grafts: a review. Exp Neurol 110:139–166.

Gage FH, Kawaja MD, Fisher LJ (1991) Genetically modified cells: applications for intracerebral grafting. Trends Neurosci 14:328–333.

Hansen JT, Koedower JH, Fiandaca MS, Jiao S-S, Notter MSD, Gash DM (1988) Adrenal medullary autografts into the basal ganglia of *Cebus* monkeys: graft viability and fine structure. Exp Brain Res 102: 65–75.

Hansen JT, Bing G, Notter MFD, Kordower JH, Fiandaca MS, Gash DM (1989) Adrenal chromaffin cells as transplants in animal models of Parkinson's disease. J Electron Microsc Tech 12:308–315.

Hawrot E, Patterson PH (1979) Long-term culture of dissociated sympathetic neurons. Methods Enzymol 58:574–584.

Herman MAR, Schulz CA, Claude P (1992) Effects of NGF and glucocorticoid on NGF receptor immunolabeling of cultured rhesus adrenal chromaffin cells. Exp Cell Res 200:370–378.

Hirsch EC, Duyckaerts C, Javoy-Agid F, Hauw JJ, Agid Y (1990) Does adrenal graft enhance recovery of dopaminergic neurons in Parkinson's disease? Ann Neurol 27:676–682.

Hurtig H, Joyce J, Sladek JR Jr, Trojanowski JQ (1989) Postmortem analysis of adrenal-medulla-to-caudate autograft in a patient with Parkinson's disease. Ann Neurol 25:607–614.

Hyman C, Hofer M, Barde Y-A, Juhasz M, Yancopoulos GD, Squinto SP, Lindsay RM (1991) BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature 350:230–232.

Jankovic J, Grossman R, Goodman C, Pirozzolo F, Schneider L, Zhu Z, Scardino P, Garber AJ, Jhingran SG, Martin S (1989) Clinical, biochemical, neuropathologic findings following transplantation of adrenal medulla to the caudate nucleus for treatment of Parkinson's disease. Neurology 39:1227–1234.

Kawaja MD, Gage FH (1991) Reactive astrocytes are substrates for the growth of adult CNS axons in the presence of elevated levels of nerve growth factors. Neuron 7:1019–1030.

Kordower JH, Fiandaca MS, Notter MFD, Hansen JT, Gash DM (1990) NGF-like trophic support from peripheral nerve for grafted rhesus adrenal chromaffin cells. J Neurosurg 73:418–428.

Kordower JH, Cochlan E, Penn RD, Goetz CG (1991) Putative chromaffin cell survival and enhanced host-derived TH-fiber innervation following a functional adrenal medulla autograft for Parkinson's disease. Ann Neurol 29:405–412.

Korsching S, Auberger G, Heunann R, Scott J, Thoenen H (1985) Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. EMBO J 4:1389–1393.

Lillien LE, Claude P (1985) Nerve growth factor is a mitogen for cultured chromaffin cells. Nature 317:632–634.

Logan A, Frautschy SA, Baird A (1991) Basic fibroblast growth factor and central nervous system injury. Ann NY Acad Sci 688:474–476.

Mahanthappa NK, Gage FH, Patterson PH (1990) Adrenal chromaffin cells as multipotential neurons for autografts. Prog Brain Res 82:33–39.

Manthorpe M, Skaper SD, Williams LR, Varon S (1986) Purification of adult rat sciatic nerve ciliary neuronotrophic factor. Brain Res 367: 282–286.

Michel RP, Cruz-Orive LM (1988) Application of the Cavalieri principle and vertical sections method to lung: estimation of volume and pleural surface area. J Microsc 150:117–136.

Niijima K, Araki M, Ogawa M, Nagatsu I, Sato F, Kimura H, Yoshida M (1990) Enhanced survival of cultured dopamine neurons by treatment with soluble extracts from chemically deafferentiated striatum of adult rat brain. Brain Res 528:151–154.

Notter MFD, Hansen JT, Okawara S, Gash DM (1989) Rodent and primate adrenal medullary cells *in vitro*: phenotypic plasticity in response to coculture with C6 glioma cells or NGF. Exp Brain Res 76: 38–46.

Olmsted JB (1986) Microtubule-associated proteins. Ann Rev Cell Biol 2:421–457.

Peters A, Palay SL, Webster HdeF (1991) The fine structure of the nervous system, 3d ed. New York: Oxford UP.

Peterson DI, Price ML, Small CS (1989) Autopsy findings in a patient who had an adrenal-to-brain transplant for Parkinson's disease. Neurology 39:235–238.

Rosenberg MB, Friedmann T, Robertson RC, Tuszynski M, Wolff JA, Breakefield XO, Gage FH (1988) Grafting genetically modified cells to the damaged brain: restorative effects of NGF expression. Science 242:1575–1578.

Schueler SB, Ortega JD, Sagen J, Kordower JH (1993) Robust survival of isolated bovine adrenal chromaffin cells following intrastriatal transplantation: a novel hypothesis of adrenal graft viability. J Neurosci 13:4496–4510.

Seidl K, Unsicker K (1989a) The determination of the adrenal medullary cell fate during embryogenesis. Dev Biol 136:481–490.

Seidl K, Unsicker K (1989b) Survival and neuritic growth of sympathoadrenal (chromaffin) precursor cells *in vitro*. Int J Dev Neurosci 7:465–473.

Stein R, Mori N, Matthews K, Lo L-C, Anderson DJ (1988a) The NGF-inducible SCG10 mRNA encodes a novel membrane-bound protein present in growth cones and abundant in developing neurons. Neuron 1:463–476.

Stein R, Orit S, Anderson DJ (1988b) The induction of a neuronal-specific gene, SCG10, by nerve growth factor in PC12 cells is transcriptional, protein synthesis dependent, glucocorticoid inhibitable. Dev Biol 127:316–325.

Sterio DC (1984) The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 134:127–136.

Stromberg I, Herrera-Marschitz M, Ungerstedt U, Ebendal T, Olson L (1985) Chronic implant of chromaffin tissue into the dopamine-denervated striatum. Effects of NGF on graft survival, fiber growth and rotational behavior. Exp Brain Res 60:335–349.

Unsicker K, Krisch B, Otten U, Thoenen H (1978) Nerve growth factor–induced fiber outgrowth from isolated rat adrenal chromaffin cells: impairment by glucocorticoids. Proc Natl Acad Sci USA 75: 3498–3502.

Weibel ER (1980) Stereological methods, Vol II. New York: Academic.

Yoshida K, Gage FH (1992) Cooperative regulation of nerve growth factor synthesis and secretion in fibroblasts and astrocytes by fibroblast growth factor and other cytokines. Brain Res 569:14–25.